Current Biology 23, 588–593, April 8, 2013 ©2013 Elsevier Ltd All rights reserved http://dx.doi.org/10.1016/j.cub.2013.03.002

Report

Numb Localizes at Endosomes and Controls the Endosomal Sorting of Notch after Asymmetric Division in *Drosophila*

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Summary

Numb acts as a cell-fate determinant during asymmetric and stem cell divisions in both vertebrates and invertebrates [1, 2]. In *Drosophila*, Numb is unequally segregated in asymmetrically dividing *s*ensory *o*rgan *p*recursor cells (SOPs). Numb is inherited by the pIIb cell (Notch OFF) and is absent from the pIIa cell (Notch ON) [3, 4]. Numb is required to establish directional Notch signaling during cytokinesis [3, 5–7]. Using real-time imaging of a functional GFP-tagged Numb, we show that Numb relocalizes during cytokinesis from the basal cortex of pIIb to subapical endosomes. This relocalization appeared to depend on its interaction with the α-adaptin [8, 9]. Live imaging of Sanpodo (Spdo), a membrane protein interacting with Numb and regulating the trafficking of Notch [6, 7, 10–15], revealed that Spdo is internalized during cytokinesis and coaccumulates with Numb in pIIb endosomes. Using a GFP-tagged Notch [6], we found that Notch coaccumulates with Spdo in a Numb-dependent manner in these pIIb endosomes. Numb was, however, dispensable for the internalization of Notch and Spdo. We propose that Numb interacts with internalized Spdo-Notch oligomers at sorting endosomes and inhibits the recycling of Notch, thereby creating an asymmetry in Notch distribution along the pIIa-pIIb interface and regulating binary fate choice.

Results and Discussion

Numb Relocalizes to Subapical Endosomes in pIIb

The localization of Numb during asymmetric cell division has so far mostly been studied on fixed tissues, using antibodies, and in living pupae, using as a proxy a GFP fused to the localization domain of Partner of Numb (Pon). Although these approaches have been useful to study the distribution of Numb at mitosis, these were not appropriate to study the dynamics of Numb localization after mitosis. We therefore generated a functional GFP-tagged Numb. A 20 kb genomic bacterial artificial chromosome (BAC) transgene [16] encoding the Numb-PA isoform fully rescued the null $numb^{15}/numb^{2}$ *trans*-heterozygous combination (Figures 1A and 1C). Because this BAC did not include the transcription start site of the *numb-RB* transcript, we conclude that the Numb-PB isoform has no essential function. We next modified this BAC by recombineering to generate NumbGFP [17]. The position of the in-frame fusion was selected based on primary sequence divergence within the *Drosophila* genus (Figure 1B). Mutant flies carrying a single copy of the NumbGFP transgene were viable and fertile with no detectable phenotype (Figure 1D), indicating that NumbGFP is fully functional. In fixed nota, NumbGFP was detected in all epithelial cells, where it localized along the basal-lateral cortex (Figures 1E and 1E′), as reported for mammalian Numb [18]. Like endogenous Numb, NumbGFP accumulated to the anterior cortex of dividing SOPs and was unequally segregated to the anterior pIIb cell (Figures 1E–1H′ and 2A–2D; see also Movie S1 available online).

We next studied the localization of Numb by live imaging. Histone2B-RFP was used to monitor mitosis and a PIP2-binding domain fused to RFP, PH-RFP, was used to mark the plasma membrane and follow the ingression of the cytokinetic furrow. Using these markers, we found that the anterior-basal crescent of NumbGFP rapidly disassembled into cortical and intracellular dots during cytokinesis (Figures 2D–2G′; Movie S1) (cytokinesis refers here to a time interval encompassing ingression of the cytokinetic furrow and extending 10 min after complete ingression). We observed that NumbGFP remained cortical during membrane blebbing (Figures 2E–E″) and formed intracellular dots clustering around the pIIb centrosome marked by Asl-RFP [19, 20] (Figures 2H–2I′). Similar NumbGFP dots were also seen around centrosomes in symmetrically dividing epidermal cells (Figures 2J and 2K). Unlike in pIIb, however, NumbGFP did not accumulate at subapical endosomes in epidermal cells. This localization of NumbGFP around centrosomes in pIIb and epidermal cells was reminiscent of the localization of Rab11 [21]. We found, however, that Numb and Rab11 localized to nonoverlapping dots around the pIIb centrosome (Figure S1). NumbGFP then accumulated at subapical endosomes in pIIb (Figures 2F–2G′). NumbGFP was first detected at this location at t = 8 ± 1 min (n = 3) (Figures 2F and F′). Moreover, endogenous Numb was found to accumulate like NumbGFP at subapical endosomes in pIIb (Figures 2L and 2L′). Finally, only low levels of NumbGFP were detected along the basal pIIa-pIIb interface during cytokinesis (Movie S1). Our analysis therefore revealed that Numb relocalizes during cytokinesis from the anterior-basal cortex to subapical endosomes in pIIb.

Sanpodo Coaccumulates with Numb in pIIb

Previous studies have shown that Spdo directly interacts with Numb and localizes in a Numb-dependent manner into pIIb endosomes [7, 10–13]. Here, we find that endogenous Spdo colocalized with Numb at subapical endosomes in pIIb (Figures 2L–2L‴). Spdo also colocalized with Rab4, Rab5, Rab7, and Rab21 [13, 15] (Figure S1), indicating that these endosomes are sorting endosomes. This suggested that Numb may regulate the endosomal sorting of Spdo. To study the dynamics of Spdo, we generated a GFP-tagged Spdo. A 20 kb genomic BAC transgene covering the *spdo* gene was found to fully rescue the null *trans*-heterozygous $spdo^{ZZ27}/spdo^{G104}$ combination (Figure 3A). Using recombineering [17], GFP was introduced at an intracellular position selected based on primary sequence divergence to generate SpdoiGFP (Spdo intracellular GFP) (Figure 3B). SpdoiGFP fully rescued the viability of $spdo^{ZZ27}/spdo^{G104}$ flies (Figure 3C), was specifically expressed in SOPs (Figure 3D) and localized similarly to

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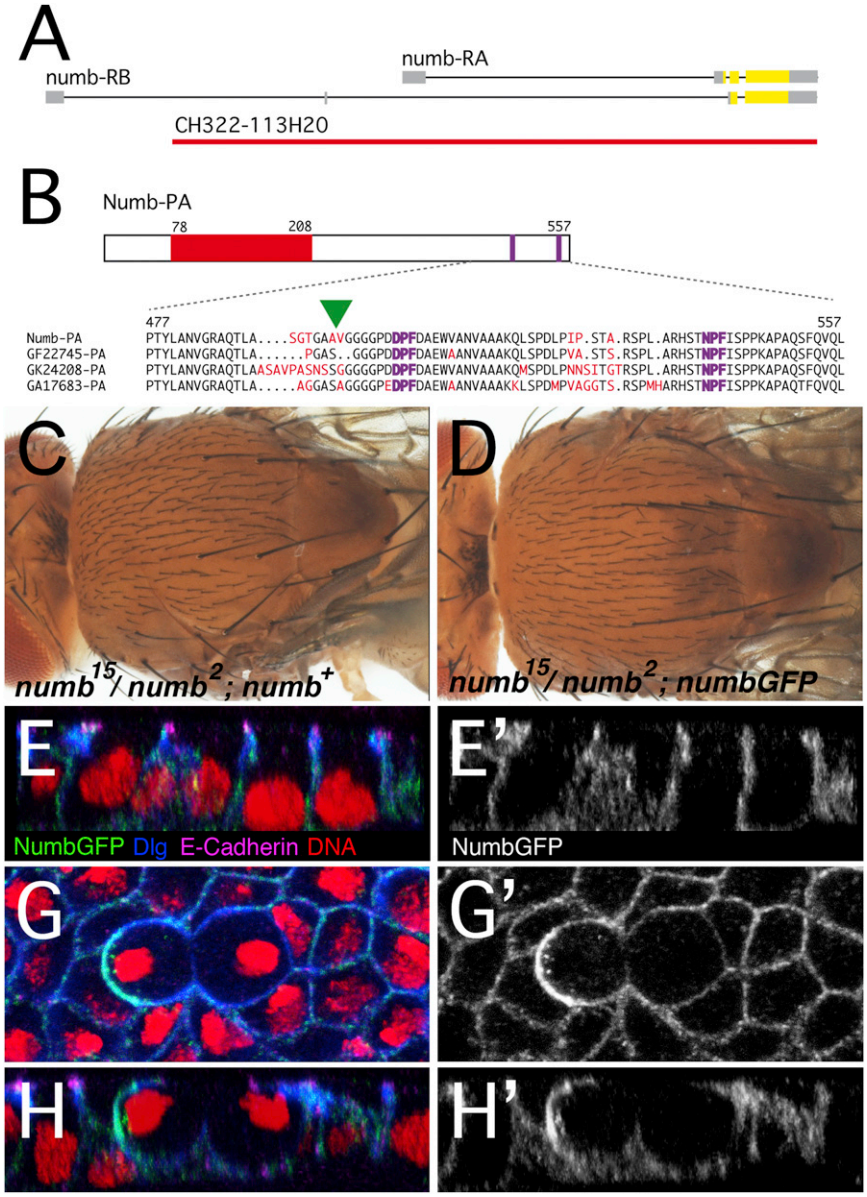

Figure 1. A Functional GFP-Tagged Numb

(A) Genomic structure of the two *numb* transcripts, RA and RB: exons are boxed; open reading frames (ORFs) are in yellow. The 20 kb BAC covering RA, but not RB, is shown in red.

(B) Structure of Numb-PA (phosphotyrosine-binding domain, red; DPF and NPF motifs, violet). GFP (green) was inserted at a position showing poor conservation between fly species.

(C and D) *numb* mutant flies rescued by one copy of the wild-type BAC (C) and the GFP-tagged BAC (D).

(E–H′) NumbGFP (green in E, G, and H; white in E′, G′, and H′) localized along the basal-lateral domain (Dlg, blue; E-Cad, violet; DAPI, red) of notum epithelial cells (E and E′). In dividing SOPs, NumbGFP was unequally segregated into the anterior pIIb cell (G–H′).

In this and all other figures, anterior is left and apical is up.

endogenous Spdo (Figure 3E). Thus, SpdoiGFP is fully functional and expressed similarly to endogenous Spdo.

In living pupae, SpdoiGFP was diffusely distributed in mitotic SOPs and equally inherited in SOP daughters (Movie S2). After division, SpdoiGFP rapidly accumulated at subapical endosomes in the pIIb cell (Figure 3F). These SpdoiGFP-positive subapical endosomes were first detected 6.1 ± 1.2 min after furrow ingression (n = 9). Thus, the kinetics of Spdo and Numb accumulation in pIIb endosomes were similar.

To test whether this endosomal accumulation of SpdoiGFP required its internalization after mitosis, we conditionally inhibited dynamin-dependent endocytosis from anaphase onward, using shi^{ts1}, a thermosensitive allele of the fly dynamin gene *shibire* (*shi*). SpdoiGFP accumulated in pIIb endosomes in shi^{ts1} pupae at permissive temperature (23°C; n = 6) and in wild-type pupae pulsed at 32°C from anaphase onward (Figure 3G) (n = 3). By contrast, SpdoiGFP accumulated along the pIIa-pIIb interface in shi^{ts1} pupae at restrictive temperature (32°C; n = 4) (Figure 3H). This demonstrated that SpdoiGFP is internalized during cytokinesis and is rapidly trafficked toward subapical endosomes in pIIb.

We next investigated the role of Numb in the endosomal accumulation of Spdo. As expected [10–13, 15], the silencing of *numb* decreased the endosomal accumulation of SpdoiGFP (Figures 3I and 3I′; Movie S3) (n = 8; see [6] for a validation of $numb^{RNAi}$). Only small SpdoiGFP-containing endosomes were observed 10 min after ingression of the furrow in both daughter cells. This suggested that Numb is not essential for the internalization of Spdo but is required for its endosomal localization. Conversely, NumbGFP was first detected at t = 9 ± 2 min in $spdo^{RNAi}$ pupae (Movie S4; Figure S2) (n = 15; see [6] for a validation of $spdo^{RNAi}$), showing that *spdo* is not essential for the relocalization of NumbGFP at subapical endosomes. However, lower levels of endosomal NumbGFP were detected in $spdo^{RNAi}$ pupae, indicating that the endosomal accumulation of Numb may involve its interaction with Spdo. We conclude that Spdo is internalized after mitosis to accumulate in a Numb-dependent manner at subapical endosomes in pIIb, suggesting that Numb regulates the postinternalization sorting of Spdo.

Notch Coaccumulates with Numb at pIIb Endosomes

We have previously shown that Spdo regulates the endocytosis of Notch [6]. Because Spdo interacts with Notch and Numb [10, 15], it may act as a linker between Notch and Numb, raising the possibility that Numb promotes the endosomal accumulation of Spdo-Notch oligomers in pIIb. To test this, we studied the distribution of Notch during cytokinesis by live imaging, using a functional GFP-tagged Notch receptor, NiGFP [6]. We found that NiGFP accumulated at subapical endosomes in pIIb, but not in pIIa (Figures 4A–4A″; Movie S5). NiGFP dots were first detected at t = 7.4 ± 1.0 min after furrow

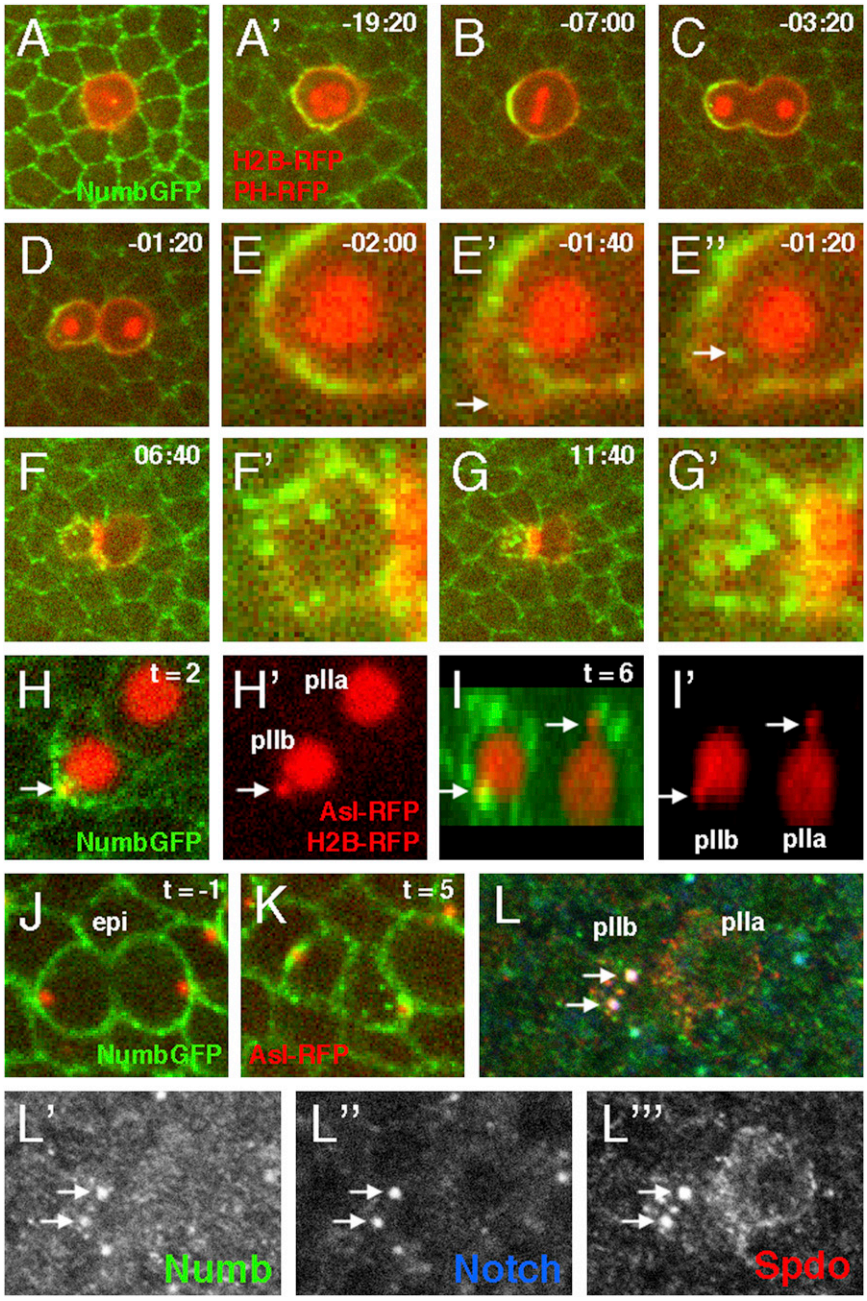

Figure 2. Live Imaging of Numb in pIIb

(A–G′) Snapshots of NumbGFP (green) in a dividing SOP marked by H2B-RFP (red) and PH-RFP (red; time in min:s). Surface (A) and basal (A′) views show the onset of basal asymmetric localization of NumbGFP at prophase. NumbGFP formed an anterior crescent at metaphase (B) and segregated into pIIb at telophase (C). NumbGFP dots were detected from telophase onward (C and D). During membrane blebbing (E–E″; arrow in E′), NumbGFP remained cortical (E′ and E″; arrow in E″). Subapical dots of NumbGFP were detected during cytokinesis (F–G′; enlarged views in F′ and G′).

(H–I′) Snapshots of NumbGFP (green) and Asl-RFP (red) in a dividing SOP (H2B-RFP, red) at t = 2 (H and H′; confocal section; t = 0, chromatin decondensation) and t = 6 (I and I′; cross-section view). Basal NumbGFP dots clustered around the pIIb centrosome.

(J and K) Snapshots of NumbGFP (green) and Asl-RFP (red) in a dividing epidermal cell at t = −1 and 5. NumbGFP dots clustered around both centrosomes.

(L–L‴) Numb (green) colocalized with Notch (blue) and Spdo (red) at subapical endosomes in pIIb.

See also Figure S1 and Movie S1.

ingression (n = 13), and at t = 10 min, 2.7 ± 1.2 NiGFP-positive dots were detected in pIIb. Endogenous Notch similarly accumulated at subapical endosomes in pIIb where it colocalized with Numb and Spdo (Figures 2L–2L‴; see also [13]).The silencing of *numb* reduced the number of these NiGFP-containing endosomes (0.2 ± 0.3 in the anterior daughter cell at t = 10 min) (Figure 4B). This correlated with increased Notch at the basal pIIa-pIIb interface [6]. The endosomal localization of NiGFP in pIIb also required the activity of Spdo: 0.6 ± 0.5 NiGFP-containing endosomes were detected in the anterior daughter cell at t = 10 min upon *spdo* silencing (Figure 4B). We therefore conclude that Notch accumulates in a Numb- and Spdo-dependent manner into subapical endosomes in pIIb during cytokinesis. This is, to our knowledge, the first direct evidence that Numb regulates the endosomal accumulation of Notch in the context of asymmetric cell division.

Numb Is Dispensable for the Internalization of Notch and Sanpodo

Although our data suggest that Numb regulates the endosomal sorting of Notch, they do not exclude the possibility that Numb also acts at the plasma membrane to promote the internalization of Notch. To test the role of Numb in the internalization of Notch and Spdo, we used a double-antibody uptake assay [6]. Internalized Notch (iNotch) was monitored via the uptake of an anti-NECD antibody, whereas internalized Spdo (iSpdo) was detected using an anti-RFP antibody in SOPs expressing Spdo-CherryL2 [22]. In this assay, iNotch and iSpdo were detected in both control and *numb* mutant SOPs, pIIa and pIIb cells (Figures 4C–4D′). We conclude that Numb is not essential for the internalization of Notch, Spdo, or Spdo-Notch complexes.

This was surprising because Numb directly interacts with α-adaptin (α-Ada), a subunit of the AP-2 complex, via its ear domain and because deletion of this domain in *ada*^{ear} mutants altered the endocytosis of Spdo and resulted in a *numb*-like phenotype [8, 15]. Interestingly, the role of this interaction is context-dependent, suggesting that it may not be central for the activity of Numb [23]. We therefore examined the role of this interaction for the internalization of Notch and found that Notch was similarly internalized in wild-type and *ada*^{ear4} mutant cells (Figure S3). However, Notch and Spdo failed to accumulate in endosomes of *ada*^{ear4} mutant pIIb cells (Figure S3; see also [15]). We conclude that the Numb-α-Ada interaction is

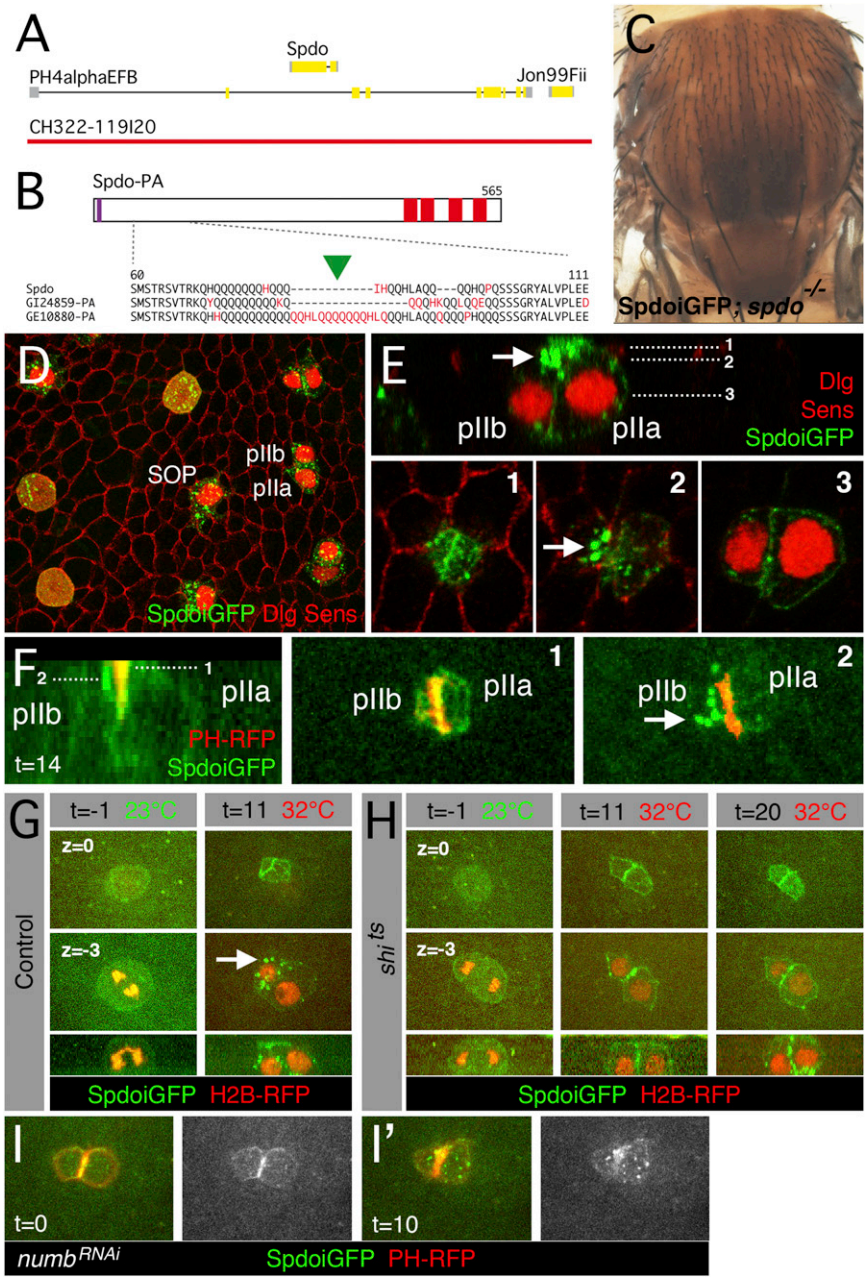

Figure 3. Dynamics of Spdo Localization in pIIb Endosomes

(A) Genomic structure of the *spdo* locus: exons are boxed; ORFs are in yellow. The rescuing BAC is in red.

(B) Domain structure of Spdo (transmembrane domains, red; NPAF motif, violet). GFP (green) was inserted at a position showing sequence variations between different fly species.

(C) *spdo* mutant fly rescued by SpdoiGFP.

(D) SOP-specific expression of SpdoiGFP (anti-GFP, green; Sens and Dlg, red) in a 16.5 hr APF notum.

(E) Cross-section and confocal (1–3, indicated by dashed lines) views showing SpdoiGFP (anti-GFP, green; Sens and Dlg, red) accumulating at subapical endosomes (arrow) in pIIb and at the plasma membrane in pIIa.

(F) SpdoiGFP (green; PH-RFP, red) localized at subapical endosomes in pIIb (arrow) at t = 14 (t = 0, complete furrow ingression). Dashed lines (1 and 2) indicate the z position of the confocal sections (right panels).

(G and H) Snapshots of SpdoiGFP (green) in control (G) and *shi*^{ts} (H) pupae shifted from 23°C to 32°C at late anaphase (t = 0; H2B-RFP, red). SpdoiGFP localized at pIIb endosomes in control (arrow; t = 11), but not *shi*^{ts} pupae. Two z sections (0 and −3 μm) and cross-section views are shown.

(I and I′) Snapshots of SpdoiGFP (green) and PH-RFP (red) at t = 0 (I; complete furrow ingression) and t = 10 (I′) in *numb*^{RNAi} pupae. SpdoiGFP localized to small subapical endosomes in both daughter cells at t = 10.

See also Figure S2 and Movies S2, S3, and S4.

important for the endosomal accumulation of Notch and Spdo, but not for the internalization of Notch. Of note, Cotton et al. have shown, using a null allele of the α-*ada* gene, that the internalization of Spdo depends on the activity of the AP-2 complex (see the accompanying paper by Cotton et al. in this issue of *Current Biology* [24]). Although we have not examined the internalization of Spdo in *ada*^{ear4} mutant cells, in which AP-2 is still partly active [8], we speculate that Spdo, like Notch, may still be internalized upon disruption of the Numb-α-Ada interaction, because it is Numb-independent. Finally, analysis of Numb localization revealed that its cortical distribution was altered in *ada*^{ear4} mutant cells (Figures 4E and 4E′). Also, though the unequal segregation of NumbGFP was not affected [8] (Figure 4F), no NumbGFP-positive endosomes were detected after division (n = 14) (Figure 4F′–4F‴) suggesting that the Numb-α-Ada interaction promotes the rapid relocalization of Numb to pIIb endosomes. Thus, the *numb*-like phenotype seen in *ada*^{ear4} mutant clones correlated with defects in Numb localization. This interpretation of the *ada*^{ear4} mutant phenotype may explain why this interaction is required in SOPs, where directional Notch signaling is established rapidly, but not in type II neuroblasts [23], where Notch-mediated decisions take longer, allowing for plasticity.

Conclusions

In summary, our results do not favor an "internalization model," whereby Numb would act at the plasma membrane of pIIb to promote the internalization of Notch (or Notch-Spdo oligomers) but rather favor a "recycling inhibition model," whereby Numb acts at sorting endosomes to inhibit the recycling of Notch-Spdo oligomers (Figure 4G). First, Notch and Spdo rapidly co-accumulate at subapical endosomes in a Numb-dependent manner. Second, this accumulation is concomitant with the rapid and Spdo-independent relocalization of Numb from the anterior-basal cortex of pIIb to these subapical endosomes. Third, the internalization of Notch and Spdo does not depend on Numb. We propose that Spdo positively regulates the endocytic turnover of Notch and that Numb inhibits the recycling of internalized Notch-Spdo oligomers via its interaction with Spdo in sorting endosomes. A similar model was proposed by Cotton et al. based on complementary data [24]. How Numb inhibits the recycling of Notch-Spdo oligomers is not

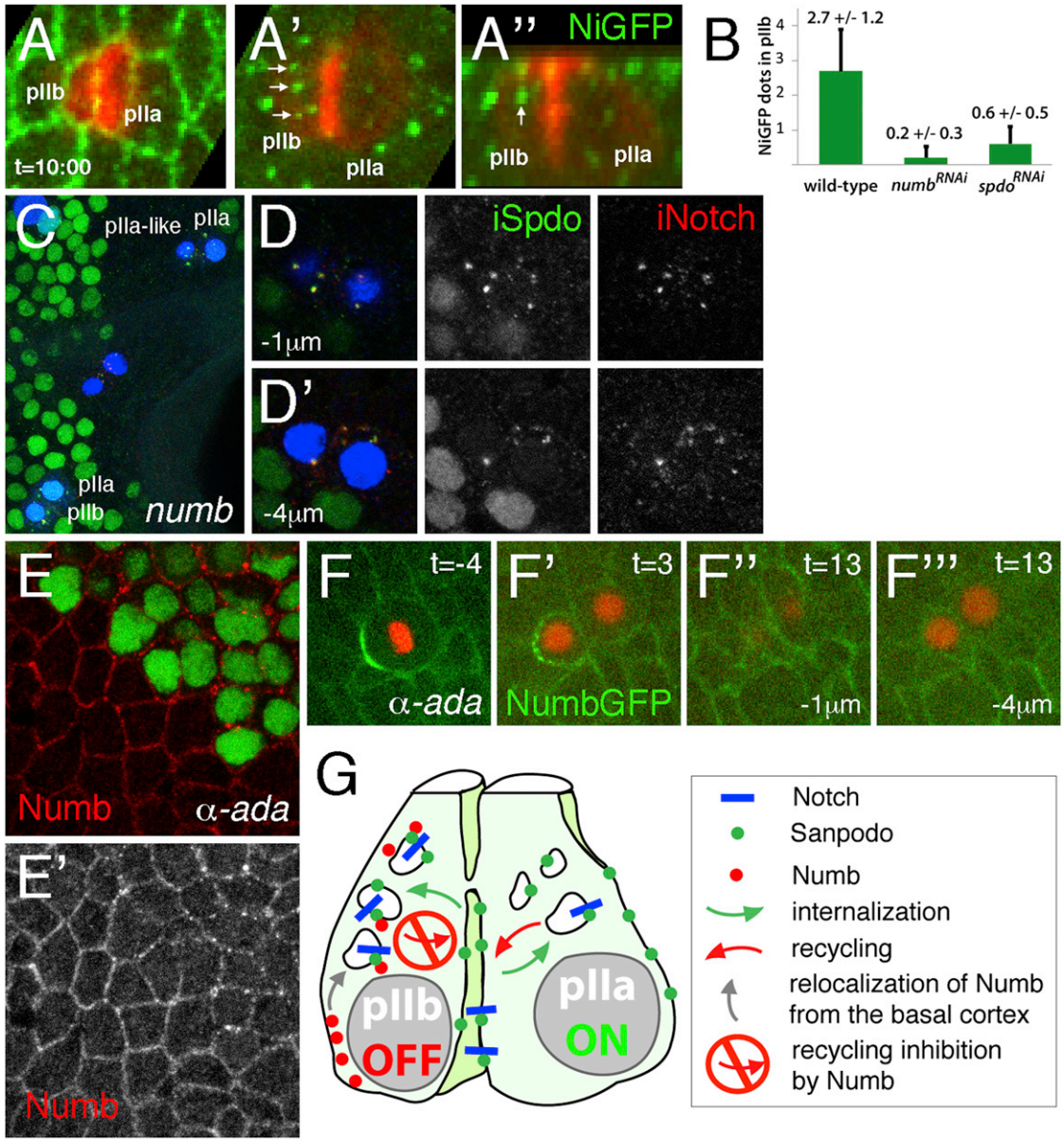

Figure 4. Numb Regulates the Endosomal Sorting of Notch

(A–A″) Snapshots from Movie S4 showing NiGFP (green) localizing into subapical endosomes (arrows) in pIIb at t = 10 min (t = 0 corresponds to complete furrow ingression; PH-RFP, red). Surface (A), subapical (A′; 1 μm below the surface), and cross-section (A″) views are shown.

(B) Quantification of the number of NiGFP-positive dots in wild-type (n = 13), $numb^{RNAi}$ (n = 10), and $spdo^{RNAi}$ (n = 12) "pIIb" cells.

(C–D′) iSpdo (green) and iNotch (red) were detected in SOP progeny cells (Sens, blue) in $numb^{2}$ mutant (marked by the absence of nuclear GFP, green) and control cells. Subapical and basal high-magnification views are shown in (B) and (B′).

(E and E′) Numb (red) localized in cortical dots in control cells (nuclear GFP, green) but displayed a smooth distribution along the basal cortex of ada^{ear4} mutant cells.

(F–F‴) Snapshots showing the distribution of NumbGFP (green) in a dividing ada^{ear4} mutant SOP (H2B-RFP, red; marked by the loss of nuclear GFP). Smooth cortical distribution was seen at telophase (F′). No subapical dots were detected during cytokinesis (t = 0, chromatin decondensation; F″ and F‴).

(G) Model of the inhibition by Numb of the recycling of Notch-Spdo oligomers.

See also Figure S3 and Movie S5.

clear. Because AP-1 positively regulates the basal-lateral targeting of both Notch and Spdo [22], one possibility is that this interaction masks a recycling signal for AP-1. Another nonexclusive possibility is that Numb links the E3 ubiquitin ligase Su(dx)/Itch to Notch [25], thereby promoting the sorting of Notch toward lysosomes [26–28]. Our recycling inhibition model is consistent with the role of Numb in nematodes [29], as well as with a previous study showing that Numb inhibits the recycling of Notch and promotes its degradation [27]. Given that a four-pass transmembrane similar to Spdo has recently been shown to interact with Numb via a conserved NPAF motif and to act antagonistically to Numb in the regulation of integrin trafficking in fish [30], this recycling inhibition model may be of general relevance.

Supplemental Information

Supplemental Information includes three figures, Supplemental Experimental Procedures, and five movies and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2013.03.002.

Acknowledgments

We thank S. Aerts, H. Bellen, P. Bryant, J. Knoblich, R. Le Borgne, J. Skeath, R. Ueda, the Bloomington Drosophila Stock Center, the Vienna Drosophila RNAi Center, the Developmental Studies Hybridoma Bank, and FlyBase for flies, antibodies, DNA, and other resources. We thank H. Rouault for the temperature-controlled stage. We are grateful to R. Le Borgne for sharing unpublished data and for scientific discussions. We thank I. Leroux and all lab members for discussion and critical reading. This work was funded by core funding from the Institut Pasteur and the Centre National de la Recherche Scientifique (CNRS) and by grants from the Fondation pour la Recherche Médicale (DEQ20100318284) and the Agence Nationale pour la Recherche (LabEx Revive, ANR-10-LABX-73).

Received: December 13, 2012
Revised: February 4, 2013
Accepted: March 1, 2013
Published: March 21, 2013

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